Manuscript EMBO-2010-74730

Molecular mechanism of Ena/VASP-mediated actin filaments elongation

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Review timeline:

Submission date:	10 May 2010
1st Editorial Decision:	17 May 2010
Appeal request	18 May 2010
Editor's reply	25 May 2010
Appeal submitted:	25 May 2010
2nd Editorial Decision:	01 July 2010
1st Revision received:	30 September 2010
3rd Editorial Decision:	02 November 2010
2nd Revision received:	05 November 2010
4th Editorial Decision:	29 November 2010
3rd Revision received:	01 December 2010
Accepted:	02 December 2010

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   17 May 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. After some delay due to the high number of manuscripts we have received over the last few weeks, I have now had the opportunity to read it carefully and to discuss it with the other members of our editorial team. I am afraid that the outcome of these discussions is not a positive one.

We appreciate your comprehensive structure/function analysis of the molecular determinants of VASP-dependent actin filament elongation. You were able to provide evidence that the rate of VASP-mediated actin filament elongation is primarily determined by the affinity of the conserved WH2-like motif for G-actin and that the elongation rate is proportional to the G-actin affinity of the WH2 motif - different WH2 motifs were tested in the context of the VASP backbone. Furthermore the present data in combination with the known actin monomer concentration within cells suggest that Ena/VASP proteins are fully saturated with actin and thus exhibit a high filament elongation rate in vivo in all species. However, you have published already that in principle VASP can mediate (processive) actin filament elongation via a mode of action that involves its WH2 domain. Clearly, we recognise that here you were able to explain the different in vitro filament elongation efficiency of VASP proteins from different organisms, to put forward a common evolutionarily conserved mechanism that is based on G-actin affinity and thus to provide some deeper insight into the mode of action of VASP in filament elongation. The study could thus potentially be of quite some interest

to readers in your field. Still, if one looks at the study from a more general conceptual point of view we do not think that the study puts forward a level of novel biological insight that is sufficiently significant to appeal to a broader molecular biology audience and thus to justify publication in The EMBO Journal. We have therefore decided not to send out the paper for in-depth peer review at this point.

Please note that we publish only a small percentage of the many manuscripts that we receive at The EMBO Journal, and that we can only subject those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. As in our carefully considered opinion, this is not the case for the present submission I am afraid to say that our conclusion regarding its publication here cannot be a positive one.

Thank you in any case for the opportunity to consider this manuscript. I am sorry that we cannot be more positive on this occasion.

Yours sincerely,

Editor
The EMBO Journal

Appeal   18 May 2010

Thank you for considering our manuscript entitled "Affinitybased mechanism of fast Ena/VASPmediated actin filament elongation". I have to admit that we were somewhat surprised that you decided not to send our manuscript for review, in particular, because the presentation of some of these data already caused enthusiastic responses at the recent ASCB meeting in San Diego. A number of topranking experts in the field suggested to submit this work to EMBO Journal.   The question of how Ena/VASP proteins modulate actin dynamics is a hot and topical theme highlighted by several review recent articles e.g. in Chesarone and Goode, 2009, Curr Opin Cell Biol., 21:2837; Insall and Machesky, 2009, Dev Cell., 17:310322, and Dominguez, 2010, Curr Opin Struct Biol., 20:217225.

Ena/VASP proteins are pivotal players in the machineries driving actinbased motility of both cells and pathogens, so a better understanding of their function in filament elongation will contribute generally to our understanding of these processes. Our conclusion that all Ena/VASP family members are equally potent filament elongators in vivo will cause waves of interest in the field.

Editor's reply   25 May 2010

Thank you for your letter asking us to reconsider our decision on your manuscript. I have now consulted with an external editorial advisor of suitable expertise who knows both the journal and the field very well. We have no objection to seeking advice from referees; but I am sure you appreciate that we cannot predict the outcome of the reviewing process. I will be in touch again as soon as I hear back from the referees.

Many thanks for your patience.

Yours sincerely,

Editor
The EMBO Journal

2nd Editorial Decision   01 July 2010

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Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. Unfortunately, the referees were not able to return their reports as quickly as initially expected.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see while referee 1 expresses concerns regarding the overall conceptual advance provided by this study and is not in favour of publication of the study here the other referees consider the study as significant and important. Still, referee 2 raises a number of major concerns regarding the conclusiveness of the data, in particular in respect to the design of the actin monomer binding experiment and the interpretation of the polymerisation data. On balance and given the strong positive vote by referees 2 and 3 I have come to the conclusion that we should be able to consider a revised manuscript in which you need to address the referees' criticisms in an adequate manner and to their satisfaction. However it will be indispensable to address all the points put forward by referee 2 and to come up with a quantitative model to interpret the polymerisation data. This is particularly important as also referee 1 raises concerns regarding the conclusiveness of this piece of evidence.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This ms follows up the interesting initial studies by the same group, showing evidence for a processive activity of VASP protein in actin filament elongation, provided that VASP molecules were clustered on a solid surface. The stimulation of barbed end growth was about 6-fold for Dictyostelium VASP, only 1.5-fold for human VASP. It was proposed that the two WH2 domains of VASP in EVH2 domain, called GAB and FAB, were involved in the processivity. Those data shed some light into unexplained effects of VASP in motility, such as the strong stimulation of actin-based movement by mammalian VASP at the lamellipodium tip and at the surface of Listeria or of ActA-coated beads, however no molecular mechanism was established. The reason why human VASP strongly enhanced velocity while it displayed poor stimulation of filament growth in in vitro assays was not clear. The present work aims at providing insight into the molecular mechanism of VASP.

It is shown that the first WH2 domain of Dictyostelium VASP (GAB) binds G-actin with a one order of magnitude higher affinity than human VASP. Replacement of human GAB by Dicty GAB in human VASP is sufficient to make the chimeric VASP almost as efficient as Dicty VASP in stimulating filament elongation. This result provides interesting information regarding the role of the GAB in the function of VASP, however it does not provide significant insight into the molecular mechanism responsible for processivity of VASP. How the higher G-actin sequestering activity of a

WH2 domain can enhance filament growth rate remains elusive and puzzling. The present work does not bring enough significant advance to be published in EMBO J.

Technical issues

The affinity of actin is measured for isolated GABs, it could be different for the GAB within the EVH2 and within the full length protein which are actually used in other experiments.

The spontaneous polymerization assays presented in Figure 3A are inconclusive. The observed effects of EVH2 could result from other changes than elongation parameters.

It is unclear why a truncated fragment of Tbeta 4 rather than Tbeta4 was used.

The model presented in figure 6 postulates that WH2-bound actin associates productively to barbed ends, but how does a higher affinity binding to actin allow better dissociation of WH2 from the barbed end, and subsequent elongation ?

Figure 6C : The saturation function of VASP by actin seems to be calculated here. Then the abscissa represents the concentration of free G-actin, presumably How are the concentrations of free G-actin evaluated in the different situations? If 100 μM free G-actin existed in vivo, would not all actin be sequestered and none assembled ?

Referee #2 (Remarks to the Author):

Breitsprecher et al. EMBO J: Affinity-based mechanism of fast Ena/VASPmediated actin filament elongation

This is an interesting and important paper. The experimental design is generally good, but serious problems with the actin monomer binding assays must be corrected. Although the analysis of the data appears to be reasonable on a qualitative level, the conclusions would be much stronger if the authors formulated and tested a quantitative model of their data, as done for formins. This is important, because the process involves so many overlapping reactions that it is impossible to use intuition to interpret the results.

The text is clear but very long-winded. A good editor could cut the length of the text by 50%. For example, rather than beginning the section on the bottom of page 6 with a question, just write a good topic sentence containing the result followed by a short description of Fig. 2C, namely that replacement of the HsGAB with the DdGAB alone or the DdGAB+FAB allows the Hs protein to elongate actin filaments as well as the Dd protein. Some paragraphs are way too long and some topic sentences ("we sought to determine the affinities of the respective GABs to actin") are buried in the middle of paragraphs. Again on page 10, one might replace the entire section from "Since the DdGAB bound G-actin..." to "accelerate actin filament elongation" with one sentence saying that you made chimeras with two foreign WH2 motifs, the WIP WH2 which binds monomers with high affinity according to (Chereau) and your new assay with OG-actin (Fig. 4B) and the Tß4 WH2 which has a low affinity of 5.6 μM for actin monomers (ref)."

Fig. 1 confirms the DdVASP stimulates elongation much better than human homologs. It is not obvious to me that the sequences of the GAB and FAB are homologous or related to WH2 motifs. Something more than the statement that they are homologous is necessary.

Fig. 2: This nice experiment shows that the GABs are the crucial parts of these proteins that determine the elongation rates. Part A could be eliminated or made smaller. A small subset of the micrographs is all that is necessary to illustrate the results, since part C summarizes all of the data very well and the movies are provided as supplemental materials. For example, the three graphs on the right side of part D could be superimposed and show the results even better than the micrographs.

Fig. 3: The spontaneous polymerization assay in part A is interesting data, but almost impossible to interpret quantitatively, because, as illustrated by part B, two opposing reactions (nucleation and elongation) are superimposed and cannot easily be disentangled. A much more sophisticated analysis is required to conclude "the lag phase was shortened when hEVH2 was present, indicating that this construct also nucleates new filaments in vitro." More likely, these concentrations of hEVH2 increase the rate of elongation, but owing to their lower affinity for monomers, do not inhibit nucleation like DdEVH2. Sorting out concentration-dependent effects on monomer sequestration, nucleation and elongation is challenging, but has been done for formins. Fortunately the authors have a key piece of the data to make such an analysis, the single filament assay in the supplemental materials (3A), which should be part of the main figure. This is the only unambiguous data in these experiments with high concentrations of EVH2. The rest of the data require more analysis ot interpret. With the elongation rates and a good mathematical model, it might be possible to interpret the data in part A.

Fig. 3C-E: The OG-actin fluorescence assay is a reasonable way to measure binding, but the assays have several problems and need to be redone. (i) What are the solution conditions for these assays? Are the concentrations on the x-axes the total concentrations of GAB or the free GAB? (ii) In either case, the shape of the curves indicates a problem. With a very low Kd such as 0.05 µM and a micromolar concentration of receptor (OG-actin) a plot of fluorescence vs. total GAB concentration should be a straight line up to saturation, where there would be a sharp inflection to the plateau. (iii) How is it possible to know the ratio of GAB per actin monomer from the fluorescence alone? Without this information, one really needs to take the reactions to saturation to know the shapes of these curves. Only one of these curves is close to saturation. A vastly better experimental design is to use much lower concentration of OG-actin (below the Kds) and to titrate to saturation with GAB. This design has the advantage that essentially all of the GAB is free rather than having to correct for an unknown amount of bound GAB. Since the concentration of OG-actin was higher than the Kds, I assume, but it was not stated in the methods, that the quadratic equation was used to correct for bound GAB, which is difficult with this experimental design.

If done properly it should be possible to formulate a good model (as done for formins) to explain quantitatively the elongation data rather than having to "suggest" that the "differences in the elongation properties of hVASP and DdVASP are mainly caused by different affinities of their GAB motifs for G-actin."

Fig. 4E: This is apparently a beautiful experiment, but it will only be believable once the actin monomer binding experiments are improved, as noted above. Since this is the key result, I would not bury the topic sentence "Plotting of the respective" in the middle of a paragraph. A quantitative model would allow the following statement to be elevated from a correlation to quantitative conclusion: "decreasing filament elongation rates observed earlier with excess amounts of DdEVH2 also correlated with the calculated saturation of the DdEVH2 with actin". The conclusion that "the elongation rate of processive filament growth on VASP-saturated beads in turn depends on the actin concentration and eventually approaches a maximal elongation rate at 100% saturation" must depend on not only the saturation of the EVH2 but also the concentration of the EVH2 (since it has to bind the filaments). Again a comprehensive quantitative model will reveal if this is true.

Fig. 5AB: The topic sentence "we varied the actin concentrations over the widest possible range" should not be buried in the middle of a paragraph. Again, these look like terrific experiments, but they are based on questionable affinities, as discussed above. Hopefully they will still look good after the binding experiments are done properly. I wonder if the following is true: "Notably, the elongation rates mediated by clustered hVASP DdGAB approached a maximal elongation rate of ~90 sub/sec instead of increasing linearly with the actin concentration (Figure 5A), demonstrating that the rate of VASP-mediated actin assembly is determined by the saturation of the protein with actin monomers." This is one possible interpretation, but it must be based on some unstated assumptions about EVH2 interacting with filaments, the rate of transfer of actin to the barbed end and the rate of dissociation of WH2/GAB from the barbed end of the filament (since these bound WH2's inhibit elongation). These assumptions need to be spelled out and justified.

Discussion: Generally very interesting and on the right track, pending assurance about the affinities. I would not say "previous studies reported controversial findings". My sense is that the data were correct, but that the experiments were done under different conditions. Given the complicated

interplay of parameters in this system (including solution conditions, EVH2 concentration, actin concentration, GAB affinity for actin monomers, saturation of GABs with actin, effects on elongation and possible effects on nucleation), it is not surprising that the interpretations differed. What is the evidence for "slow kinetics of monomer binding to the GAB"? I would be surprised if the rate of monomer binding to GAG were not a factor in the elongation rate (at least is for formins). A quantitative model will answer this question.

Additional comments by referee 2:

The authors really need to do an additional binding experiment, where they compete GAB/WH2 from Oregon-Green-labeled actin monomers with unlabeled actin monomers. Such a competition experiment is required to measure the affinity of GAB/WH2 for unlabeled actin, which may be different from the affinity for OG-labeled actin.

Referee #3 (Remarks to the Author):

This study investigates the mechanism by which Ena/VASP proteins enable enhanced actin filament elongation by actin monomer binding and presentation of actin to barbed ends of filaments. The authors show that the affinity of the GAB domain of Ena/VASP for G-actin dictates the saturation of the binding sites on tetrameric Ena/VASP complexes. The strength of this study is the detailed biochemical analysis of the domains of VASP proteins, and particularly the GAB domain, through the production of chimeras of fast and slow VASP proteins. This analysis leads to the generation of a model potentially encompassing all Ena/VASP elongation rate enhancement functionality. It describes how VASP enhancement of actin growth rates can be understood from the key parameters: including actin concentration, binding site occupancy, and G-actin affinity. The biochemical mechanism proposed for VASP proteins is precise and detailed, and I find it to be much more satisfying than previous models. This represents a critical advance in understanding VASP mechanism for controlling actin dynamics, which underlies many important cellular processes.

I only have a few suggestions for improving the paper:

1. To provide an even more complete picture, could the authors speculate about the nature of the differences in VASP functionality? Is there any in vivo relevance to the range of actin affinity exhibited by GAB from different organisms in terms of cell motility or biochemical function?

2. To clarify some of the finer points of the mechanism, does the differential affinity of the GAB domain for actin have any impact on the ability of VASP constructs to protect from capping proteins? Determination of the anti-capping activity of the chimeras either in solution or on functionalized bead surfaces would reveal this. Further, what role does the affinity of the FAB for F-actin play in the mechanism? And, does this affinity for F-actin change if the GAB is saturated with actin monomers? Examination of the F-actin binding affinity by co-sedimnetation in the absence of G-actin and the presence of high concentrations of latrunculin A-bound actin monomers could provide this information. This could perhaps reveal the relevance of the unique attributes of the DdVASP(FAB) and whether the dual affinity for F-actin and G-actin plays a role in the high activity of the DdVASP protein.
3. There are some places in the paper where the writing is difficult to get through, and would benefit from a round of editing for clarity, possibly with help from fresh eyes (non-authors). The mechanisms and models are detailed, so it is important to put effort into making these elegant findings accessible to the average reader, or even actin experts who are not Ena/VASP experts.

In sum, this study is a tour de force on VASP mechanism. The data are of very high quality, and the story provides important new understanding of this essential actin assembly mechanism. The work will be of great interest to the field, and is highly appropriate for EMBO J.

Referee #1 (Remarks to the Author):

*This ms follows up the interesting initial studies by the same group,
showing evidence for a processive activity of VASP protein in actin filament
elongation, provided that VASP molecules were clustered on a solid surface.
The stimulation of barbed end growth was about 6-fold for Dictyostelium
VASP, only 1.5-fold for human VASP. It was proposed that the two WH2 domains
of VASP in EVH2 domain, called GAB and FAB, were involved in the
processivity. Those data shed some light into unexplained effects of VASP in
motility, such as the strong stimulation of actin-based movement by
mammalian VASP at the lamellipodium tip and at the surface of Listeria or of
ActA-coated beads, however no molecular mechanism was established. The
reason why human VASP strongly enhanced velocity while it displayed poor
stimulation of filament growth in in vitro assays was not clear. The present
work aims at providing insight into the molecular mechanism of VASP.
It is shown that the first WH2 domain of Dictyostelium VASP (GAB) binds
G-actin with a one order of magnitude higher affinity than human VASP.
Replacement of human GAB by Dicty GAB in human VASP is sufficient to make
the chimeric VASP almost as efficient as Dicty VASP in stimulating filament
elongation. This result provides interesting information regarding the role
of the GAB in the function of VASP, however it does not provide significant
insight into the molecular mechanism responsible for processivity of VASP.
How the higher G-actin sequestering activity of a WH2 domain can enhance
filament growth rate remains elusive and puzzling. The present work does not
bring enough significant advance to be published in EMBO J.*

We now include a quantitative model of VASP-mediated filament elongation (as
suggested by reviewer 2) that provides further insight into the mechanism of
VASP-mediated filament elongation.

It is not clear what the reviewer meant by "higher G-actin sequestering activity of
a WH2 domain can enhance filament growth rate" but assume this relates to the
pyrene assays with excess of EVH2 originally presented in Figure 3. Although
these results are in line with our model, they are not required for the conclusion of
this study. To avoid confusion, these data are now replaced by a new
supplementary Figure (Supplementary Figure 4) showing the effects of the DdGAB
peptide alone on actin assembly in pyrene assays. These experiments show that
the GAB inhibits spontaneous nucleation but does not sequester monomers (as
shown for other WH2 motifs or profilin, e.g. shown in (Carlier et al, 2007, Hertzog
et al, 2002, Hertzog et al, 2004, Korenbaum et al, 1998)), indicating that it allows
the addition of monomers to the filament barbed end without capping it
afterwards. These data show the specific properties of the GAB motif much more
clearly, and are therefore more relevant to the mechanism of WH2-mediated
filament elongation.

Technical issues

*The affinity of actin is measured for isolated GABs, it could be different
for the GAB within the EVH2 and within the full length protein which are
actually used in other experiments.*

This is a good point. We therefore also measured the actin-affinity of the DdGAB
within the DdEVH2 domain for actin, and found that the KD values are virtually
identical. The data are now shown in Supplementary Figure 3.

*The spontaneous polymerization assays presented in Figure 3A are
inconclusive. The observed effects of EVH2 could result from other changes*

than elongation parameters.

It is true that many overlapping reactions (nucleation, elongation and sequestering) influence the outcome of these assays. Since these assays obviously caused some confusion, and since they are not necessary for the conclusion of our work, we decided to remove them from the manuscript. Nevertheless, it was shown by TIRF microscopy (originally presented in supplementary Figure 3) that the observed inhibitory effects at an excess of DdEVH2 are most likely caused by decreased elongation rates. This is actually in line with our model, which features a saturation-dependence of VASP-mediated filament elongation (and hence a slower elongation when less GAB within the VASP tetramer are bound to actin).

It is unclear why a truncated fragment of Tbeta 4 rather than Tbeta4 was used.

Thymosin ß4 (Tbeta4) is an actin monomer sequestering protein which is composed of an N-terminal, barbed end binding WH2 motif (which we inserted into the hVASP backbone in this study) and a C-terminal region binding to the pointed end. We used a truncated fragment of Tbeta4, since we wanted to analyze whether WH2 motifs from other proteins can mimic the GAB function. Full-length Tbeta4 efficiently sequesters actin monomers by binding to both, the barbed end (via the WH2 motif) and the pointed end (via the C-terminal helix), hence exerting an entirely different function than an isolated WH2 motif. Thus, it would not have been meaningful to insert the full-length sequence. As shown by Hertzog et al., (2004) point mutations in the C-terminal helix prevent sequestration and lead to the formation of G-actin-WH2 complexes that exclusively participate in barbed end elongation, like a typical WH2 motif, reminiscent of profilin.

The model presented in figure 6 postulates that WH2-bound actin associates productively to barbed ends, but how does a higher affinity binding to actin allow better dissociation of WH2 from the barbed end, and subsequent elongation ?

It was shown previously that WH2 motifs bind to the barbed end of the actin monomer, and therefore do not necessarily interfere with actin filament assembly at the barbed end (Chereau et al, 2005, Hertzog et al, 2002, Hertzog et al, 2004). These properties are reminiscent of profilin, which also binds actin monomers at the barbed end but does not inhibit filament elongation (Gutsche-Perelroizen et al, 1999, Korenbaum et al, 1998, Perelroizen et al, 1996). Our findings and those from other labs therefore suggest that both, profilin and WH2 motifs rapidly detach from the monomer once it is incorporated into the barbed end. Thus, a higher affinity of the WH2 motifs within the VASP tetramer would exclusively result in a higher saturation of the protein with monomers at low actin concentrations (and hence faster elongation) without negatively affecting transition of WH2-bound monomers to the barbed end. Additionally, all experimental data with hVASP chimeras bearing different WH2 motifs can be fitted with a single kt value (which describes the rate of monomer transition to the barbed end), suggesting indeed that the different monomer affinities of these motifs do not influence the rate of monomer delivery.

Figure 6C : The saturation function of VASP by actin seems to be calculated here. Then the abscissa represents the concentration of free G-actin, presumably. How are the concentrations of free G-actin evaluated in the different situations? If 100 μM free G-actin existed in vivo, would not all actin be sequestered and none assembled?

Yes, the saturation curve was indeed calculated. It is generally believed that most monomeric actin is complexed with profilin or Tbeta4 in vivo to prevent spontaneous, unproductive nucleation of filaments. However, we have shown

before that VASP can use both, actin and profilin-actin, for elongation (Breitsprecher et al, 2008), which means that at least profilin-actin complexes will be a fuel-source for VASP-mediated filament elongation. Moreover, a crystal structure of a complex of a PRD-GAB peptide and profilin actin strongly suggested that both, GAB and profilin can bind the barbed end of the monomer simultaneously (Ferron et al, 2007). The revised version of Figure 6 now shows the elongation rates calculated according to our mathematical model (see comments to Referee#2).

Referee #2 (Remarks to the Author):

This is an interesting and important paper. The experimental design is generally good, but serious problems with the actin monomer binding assays must be corrected. Although the analysis of the data appears to be reasonable on a qualitative level, the conclusions would be much stronger if the authors formulated and tested a quantitative model of their data, as done for formins. This is important, because the process involves so many overlapping reactions that it is impossible to use intuition to interpret the results.

This reviewer raised a number of very important points. First, we have repeated the monomer-binding assays as suggested and additionally performed stoppedflow experiments to analyze the kinetics of the EVH2-actin interaction (which is now presented in Figure 3).

More importantly, we agree that a testable quantitative model was needed to unravel the mechanism of VASP-mediated filament elongation. Therefore, we teamed up with Richard B. Dickinson from the University of Florida in Gainsville, FA, who has previously published several important papers on filament elongation by end-tracking proteins (Dickinson & Purich, 2002, Dickinson et al, 2002, Dickinson et al, 2004, Dickinson, 2009). With his help and based on additional experimental data, we were able to formulate a quantitative mathematical model that now explains the saturation-dependence of processive VASP-mediated filament elongation in more detail, and that moreover provides new insights into the kinetics and molecular mechanisms of the elongation process.

The text is clear but very long-winded. A good editor could cut the length of the text by 50%. For example, rather than beginning the section on the bottom of page 6 with a question, just write a good topic sentence containing the result followed by a short description of Fig. 2C, namely that replacement of the HsGAB with the DdGAB alone or the DdGAB+FAB allows the Hs protein to elongate actin filaments as well as the Dd protein. Some paragraphs are way too long and some topic sentences ("we sought to determine the affinities of the respective GABs to actin") are buried in the middle of paragraphs. Again on page 10, one might replace the entire section from "Since the DdGAB bound G-actin..." to "accelerate actin filament elongation" with one sentence saying that you made chimeras with two foreign WH2 motifs, the WIP WH2 which binds monomers with high affinity according to (Chereau) and your new assay with OG-actin (Fig. 4B) and the Tb4 WH2 which has a low affinity of 5.6 µM for actin monomers (ref)."

We followed the suggestion of the reviewer and shortened the text in the stated sections.

Fig. 1 confirms the DdVASP stimulates elongation much better than human homologs. It is not obvious to me that the sequences of the GAB and FAB are homologous or related to WH2 motifs. Something more than the statement that they are homologous is necessary.

We now added a supplementary figure (Supplementary Figure 1), which shows a sequence alignment of the GAB motifs with several WH2 motifs found in actinbinding proteins from various organisms. Since only the GAB (but not the FAB) displays high sequence similarities to WH2 motifs, we changed the wording in the manuscript, now stating that the GAB is a typical WH2 motif, and that the FAB was proposed to posses WH2-like properties (as proposed in (Dominguez, 2007, Dominguez, 2009, Dominguez, 2010).

Fig. 2: This nice experiment shows that the GABs are the crucial parts of these proteins that determine the elongation rates. Part A could be eliminated or made smaller. A small subset of the micrographs is all that is necessary to illustrate the results, since part C summarizes all of the data very well and the movies are provided as supplemental materials. For example, the three graphs on the right side of part D could be superimposed and show the results even better than the micrographs.

We followed the suggestion of the reviewer and downsized Figure 2A. However, we still feel that is important to show the TIRF-micrographs of filament elongation mediated by the VASP chimeras in the main Figure in the manuscript to illustrate that they are functional like WT proteins in solution and on beads.

Fig. 3: The spontaneous polymerization assay in part A is interesting data, but almost impossible to interpret quantitatively, because, as illustrated by part B, two opposing reactions (nucleation and elongation) are superimposed and cannot easily be disentangled. A much more sophisticated analysis is required to conclude "the lag phase was shortened when hEVH2 was present, indicating that this construct also nucleates new filaments in vitro." More likely, these concentrations of hEVH2 increase the rate of elongation, but owing to their lower affinity for monomers, do not inhibit nucleation like DdEVH2. Sorting out concentration-dependent effects on monomer sequestration, nucleation and elongation is challenging, but has been done for formins. Fortunately the authors have a key piece of the data to make such an analysis, the single filament assay in the supplemental materials (3A), which should be part of the main figure. This is the only unambiguous data in these experiments with high concentrations of EVH2. The rest of the data require more analysis to interpret. With the elongation rates and a good mathematical model, it might be possible to interpret the data in part A.

We appreciate that the pyrene assays originally presented in Figure 3 are interesting. However, in order to make the manuscript more accessible to nonexperts, we decided to remove these data from that manuscript for the following reasons: One of the major problems is indeed that the pyrene assays show many overlapping reactions and are affected by sequestering, elongation and nucleation. The latter parameter seems to be different for hEVH2 and DdEVH2. However, it was reported previously that VASP-mediated filament nucleation is negligible in vivo (Barzik et al, 2005, Bear et al, 2000, Bear et al, 2002) and therefore most likely not a critical function of Ena/VASP proteins. Since our work – and the mathematical model – focuses on the elongation properties of Ena/VASP proteins, we decided not to follow nucleation in more detail. A detailed analysis of nucleation and sequestering could be interesting, but would require including many additional parameters that would make our model more complicated without significantly clarifying the VASP-mediated elongation mechanism.

Moreover, we also think that the decrease in filament elongation by an excess of DdEVH2 in solution is an interesting finding, which however again is not required to support our conclusions and may cause more confusion than clarity. We feel that the saturation-dependence of VASP-mediated filament elongation is clearly described in Figures 4 and 5 and supported by our model. After careful consideration, we therefore decided to leave this part out.

Fig. 3C-E: The OG-actin fluorescence assay is a reasonable way to measure binding, but the assays have several problems and need to be redone. (i) What are the solution conditions for these assays? Are the concentrations on the x-axes the total concentrations of GAB or the free GAB? (ii) In either case, the shape of the curves indicates a problem. With a very low Kd such as 0.05 μM and a micromolar concentration of receptor (OG-actin) a plot of fluorescence vs. total GAB concentration should be a straight line up to saturation, where there would be a sharp inflection to the plateau. (iii) How is it possible to know the ratio of GAB per actin monomer from the fluorescence alone? Without this information, one really needs to take the reactions to saturation to know the shapes of these curves. Only one of these curves is close to saturation. A vastly better experimental design is to use much lower concentration of OG-actin (below the Kds) and to titrate to saturation with GAB. This design has the advantage that essentially all of the GAB is free rather than having to correct for an unknown amount of bound GAB. Since the concentration of OG-actin was higher than the Kds, I assume, but it was not stated in the methods, that the quadratic equation was used to correct for bound GAB, which is difficult with this experimental design.

We appreciate the importance of these assays for the conclusion of our study, and therefore repeated the fluorescence titrations as suggested by the referee. All titration curves now approach saturation more clearly. Notably, the K_D values derived from these new experiments do not vary significantly from the previous ones. Specific points:

(i) We apologize for not being clear on the buffer conditions in the figure legend and the labeling of the axes. The x-axis indeed shows the total concentration of the GAB peptide, which is now stated in the graph. The respective actin concentrations used are also given in the graphs. All reactions were carried out in G-buffer supplemented with KCl and LatA as indicated in Figures 3 A and B as well as in the Material and Methods section.

(ii) We do not see why the shape of the curve indicates a problem. As stated in the figure legend, 100 nM (and not 1 μM) OG-actin was used to analyze the binding of the DdGAB in G-buffer. We apologize if this was not stated clearly in the text. Under these conditions, fluorescence titrations yield a titration curve that is well suited for fitting. To make this clearer, we also performed an additional titration of the DdGAB peptide in G-buffer with 1 μM actin (see Figure 1 below). As illustrated, this curve is indeed (nearly) a straight line up to saturation.
Figure1: Fluorescence titration of 1 μM OG-actin monomers with the DdGAB peptide in G-buffer. The solid line represents a calculated binding isotherm, yielding a K_D of ~60 nM.
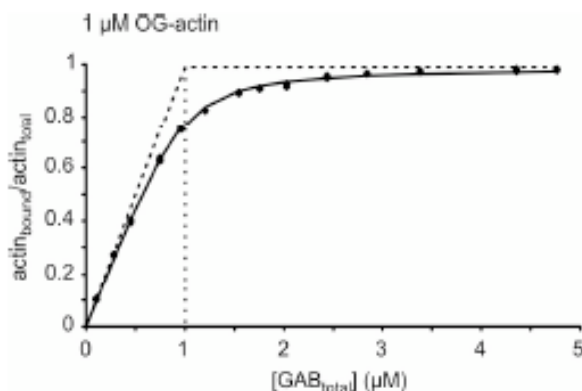
(iii) We indeed used the quadratic equation for a simple bimolecular interaction to obtain these data. For clarity, the equation is now given in the Material and Methods section.

If done properly it should be possible to formulate a good model (as done for formins) to explain quantitatively the elongation data rather than having to "suggest" that the "differences in the elongation properties of hVASP and DdVASP are mainly caused by different affinities of their GAB motifs for G-actin."

As stated above, we developed a mathematical model on the basis of the actoclampin model of filament elongators. The model is now presented in the new Figure 5 and described in more detail in the main text, the Material and Methods section and in the Supplementary information.

Fig. 4E: This is apparently a beautiful experiment, but it will only be believable once the actin monomer binding experiments are improved, as noted above.

We repeated the experiments as suggested by the reviewer, and the obtained KD values were virtually identical to those derived from prior experiments.

Since this is the key result, I would not bury the topic sentence "Plotting of the respective" in the middle of a paragraph. A quantitative model would allow the following statement to be elevated from a correlation to quantitative conclusion: "decreasing filament elongation rates observed earlier with excess amounts of DdEVH2 also correlated with the calculated saturation of the DdEVH2 with actin".

The reviewer is right that these findings can also be explained by our saturation-based elongation mechanism. However, as stated above, we decided to remove the data showing the decrease in filament elongation rates with an excess of DdEVH2 in solution from the manuscript for reasons of clarity.

The conclusion that "the elongation rate of processive filament growth on VASP-saturated beads in turn depends on the actin concentration and eventually approaches a maximal elongation rate at 100% saturation" must depend on not only the saturation of the EVH2 but also the concentration of the EVH2 (since it has to bind the filaments). Again a comprehensive quantitative model will reveal if this is true.

We are not quite sure what is meant with "concentration of the EVH2", since VASP is clustered on a surface. The only parameter that can be determined for such a setup is the coating density. We have shown previously that processive, VASPmediated filament elongation requires dense clustering of the proteins (Breitsprecher et al., 2008). However, our model also implies that a fixed number of GAB sites mediates filament elongation at a time.

Fig. 5AB: The topic sentence "we varied the actin concentrations over the widest possible range" should not be buried in the middle of a paragraph. Again, these look like terrific experiments, but they are based on questionable affinities, as discussed above. Hopefully they will still look good after the binding experiments are done properly. I wonder if the following is true: "Notably, the elongation rates mediated by clustered hVASP DdGAB approached a maximal elongation rate of 90 sub/sec instead of increasing linearly with the actin concentration (Figure 5A), demonstrating that the rate of VASP-mediated actin assembly is determined by the saturation of the protein with actin monomers." This is one possible interpretation, but it must be based on some unstated assumptions about EVH2 interacting with filaments, the rate of transfer of actin to the barbed end

*and the rate of dissociation of WH2/GAB from the barbed end of the filament
(since these bound WH2's inhibit elongation). These assumptions need to be
spelled out and justified.*

We now analyze these data in much more detail by applying our model. We were
able to confirm that the elongation rate mediated by VASP on the bead surface is
likely to approach a maximal elongation rate once the protein is saturated with
monomers. With this much more sophisticated approach, we found that the
predicted maximal rate mediated by VASP is higher than previously assumed
(~125 sub/sec). On the basis of our model and the new stopped-flow data, we were
moreover able to derive values for the transfer-rate of GAB-bound monomers to
the tip (kt). Importantly, all data can be well fitted with an identical kt-value,
suggesting that inhibition of filament elongation by the different WH2 motifs is
negligible during VASP-mediated filament elongation.

*Discussion: Generally very interesting and on the right track, pending
assurance about the affinities. I would not say "previous studies reported
controversial findings". My sense is that the data were correct, but that
the experiments were done under different conditions. Given the complicated
interplay of parameters in this system (including solution conditions, EVH2
concentration, actin concentration, GAB affinity for actin monomers,
saturation of GABs with actin, effects on elongation and possible effects on
nucleation), it is not surprising that the interpretations differed.*

We did not intend to state that the findings were incorrect. To prevent
misinterpretation, we changed the wording and now state that many findings were
incoherent due to different buffer conditions and varying actin/VASP
concentrations in a number of studies.

*What is the evidence for "slow kinetics of monomer binding to the GAB"? I would be
surprised if the rate of monomer binding to GAB were not a factor in the
elongation rate (at least is for formins). A quantitative model will answer
this question.*

The reviewer was right. Our model and the analysis of the binding kinetics of actin
to the EVH2 domains by stopped-flow measurements revealed that the on-rate of
monomer binding is indeed a factor affecting the elongation rate. Therefore, we
removed the assumptions on the binding kinetics from the manuscript, and now
present experimental and calculated kinetic data.

additional comments by referee 2:

*The authors really need to do an additional binding experiment, where
they compete GAB/WH2 from Oregon-Green-labeled actin monomers with
unlabeled actin monomers. Such a competition experiment is required
to measure the affinity of GAB/WH2 for unlabeled actin, which may be
different from the affinity for OG-labeled actin.*

We performed competition experiments with unlabeled actin, and found that the
peptides bound to labeled actin with slightly higher affinity (1.7-2 fold) compared
to unlabeled actin, which is now stated in the manuscript. In a second approach,
we analyzed the assembly of OG-labeled actin into filaments on the surface of
hVASP- and DdVASP-coated beads in the absence of CP. This way, one can
distinguish between spontaneously growing and VASP-assembled actin filaments
(Breitsprecher et al., 2008). It was previously shown for formins that the
fluorescence intensity of formin-assembled filaments was significantly lower
when profilin was present due to the drastically lower affinity of profilin to labeled
monomers. If the GABs within the VASP tetramer would display dramatically
different affinities for labeled and unlabeled actin, one would expect different
fluorescence intensities of the filaments formed. However, we did not detect

differences in the fluorescence intensity of VASP-assembled filaments growing with their barbed end attached to bead surface and filaments growing with free barbed ends, validating that there is no striking difference in the binding properties of the GAB motifs to labeled and unlabeled actin (see Figure 2 below).
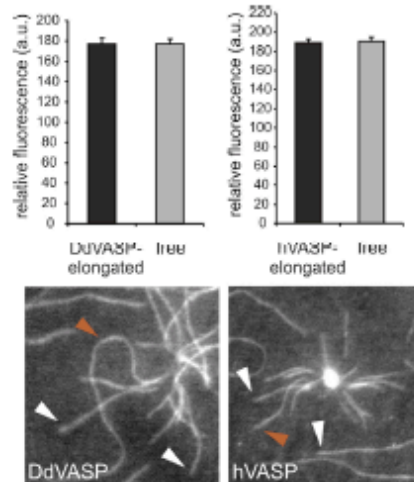
Figure 2: Comparison of fluorescence intensities of actin filaments assembled either spontaneously or by VASP. The mean fluorescence intensities of the actin filaments were determined with the line-scan tool implemented in the ImageJ software. Red arrowheads indicate VASP-assembled filaments, white arrowheads indicate spontaneously growing filament ends. n ≥ 15 for each experiment.

Referee #3 (Remarks to the Author):

This study investigates the mechanism by which Ena/VASP proteins enable enhanced actin filament elongation by actin monomer binding and presentation of actin to barbed ends of filaments. The authors show that the affinity of the GAB domain of Ena/VASP for G-actin dictates the saturation of the binding sites on tetrameric Ena/VASP complexes. The strength of this study is the detailed biochemical analysis of the domains of VASP proteins, and particularly the GAB domain, through the production of chimeras of fast and slow VASP proteins. This analysis leads to the generation of a model potentially encompassing all Ena/VASP elongation rate enhancement functionality. It describes how VASP enhancement of actin growth rates can be understood from the key parameters: including actin concentration, binding site occupancy, and G-actin affinity. The biochemical mechanism proposed for VASP proteins is precise and detailed, and I find it to be much more satisfying than previous models. This represents a critical advance in understanding VASP mechanism for controlling actin dynamics, which underlies many important cellular processes.

I only have a few suggestions for improving the paper:

1. To provide an even more complete picture, could the a uthors speculate about the nature of the differences in VASP functionality? Is there any in vivo relevance to the range of actin affinity exhibited by GAB from different organisms in terms of cell motility or biochemical function?

At the current point, it is difficult to speculate why the actin affinities vary that much. It might well be that the actin monomer pool within the cell is funneled into

different processes by competition of actin binding proteins for monomers. For example, it is conceivable that Dictyostelium amoeba employ their high-affinity VASP protein to preferentially funnel monomers for filament elongation and hence cell migration (since rapid migration is key for the life-cycle of this organism). In vertebrates, the situation might be more balanced by a lower affinity VASP, leaving a larger amount of monomers for other actin-binding proteins. Such a conclusion would however require a comparison of affinities and protein levels of a variety of actin binding proteins in the different cell types. Therefore, we prefer not to speculate on a possible in vivo relevance of these differences.

2. To clarify some of the finer points of the mechanism, does the differential affinity of the GAB domain for actin have any impact on the ability of VASP constructs to protect from capping proteins? Determination of the anti-capping activity of the chimeras either in solution or on functionalized bead surfaces would reveal this.

We aimed to find differences in the capping protein resistance of processive filament elongation mediated by hVASP, DdVASP, and the chimeric proteins on coated beads. We employed TIRF microscopy with varying Cap32/34 and CapZ concentrations, and did not find a concentration-dependent effect on filament assembly by Ena/VASP in the presence of CP concentrations up to 1 μM. Additionally, we performed pyrene-assays with coated beads and titrated capping protein but again failed to detect any differences. Thus, it seems unlikely that differences in the G-actin affinities influence the CP resistance of Ena/VASP proteins, which in turn again strongly suggests that VASP-elongated filaments on a surface are indeed resistant to CP (as stated in Breitsprecher et al.,2008).

Further, what role does the affinity of the FAB for F-actin play in the mechanism? And, does this affinity for F-actin change if the GAB is saturated with actin monomers? Examination of the F-actin binding affinity by co-sedimnetation in the absence of G-actin and the presence of high concentrations of latrunculin A-bound actin monomers could provide this information. This could perhaps reveal the relevance of the unique attributes of the DdVASP(FAB) and whether the dual affinity for F-actin and G-actin plays a role in the high activity of the DdVASP protein.

This is a very good point. As recommended by the referee, we performed highspeed sedimentation assays with DdVASP and hVASP. We polymerized actin in the presence of different concentrations of VASP and analyzed pellets and supernatants by SDS-PAGE. From these assays, we estimated that DdVASP bound to F-actin with a significantly lower affinity than hVASP, with KDs of roughly 1 μM for DdVASP and 100 nM for hVASP. When adding the same concentrations of VASP to pre-polymerized F-actin, we found that the KDs were insignificantly lower, suggesting that there is no difference in F-actin binding with monomers present or absent in the solution.

Our finding that DdVASP bound to F-actin much weaker than hVASP, and that the insertion of the DdFAB into hVASP was already sufficient to cause an increase in the filament elongation rates suggested that lower binding affinities of the DdFAB may enhance the rate of monomer transfer to the barbed end. This would follow if monomer recruitment by the adjacent GAB was only possible when the FAB is detached from the filament. Thus, a shorter dwell-time of the DdFAB at the side of the filament would result in an increase of the monomer transfer rate, which is now stated in the discussion. Since this is relevant to an understanding of the mechanism of VASP-mediated filament elongation we include these data as a separate Figure (Figure 7).

3. There are some places in the paper where the writing is difficult to get through, and would benefit from a round of editing for clarity, possibly with help from fresh eyes (non-authors). The mechanisms and models are

*detailed, so it is important to put effort into making these elegant
findings accessible to the average reader, or even actin experts who are not
Ena/VASP experts.*

The manuscript is now considerably revised and hopefully clearer. As stated
above, we removed some of the confusing data originally presented in Figure 3
and now present a testable model describing the elongation mechanism, which
will make the manuscript more accessible for non-experts.

*In sum, this study is a tour de force on VASP mechanism. The data are of
very high quality, and the story provides important new understanding of
this essential actin assembly mechanism. The work will be of great interest
to the field, and is highly appropriate for EMBO J.*

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3rd Editorial Decision	02 November 2010

Thank you for sending us your revised manuscript. Our original referee 2 has now seen it again; and he/she is now more positive about publication of the paper here. Still, in addition to a number of minor points he/she has certain issues with the completeness of the newly added mathematical model and its validation that in his/her view still need to be addressed (see below) before we can ultimately accept your manuscript. I would therefore like to ask you to deal with the issues raised in an amended version of the manuscript. Please let us have a suitably re-revised version of the manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

Breitsprecher et al. EMBO J revised

The authors responded very constructively to the first round of review, conducted new experiments, added a mathematic analysis and rewrote parts of the paper. Overall, the revised ms is far better than the original. Some of the new parts of the paper still need some refinement.

p. 5, final paragraph of introduction: After explaining some fundamental differences between formins and VASP, the final conclusion regarding the mechanism for delivery of actin subunits to the barbed end of filaments is remarkably like the formin mechanism, i.e. rate-limiting binding of subunits from solution to multiple sites on the protein (formin or VASP) followed by rapid transfer to the barbed end and rapid dissociation of the protein that delivered the new subunit. This might be noted. But then I would say that compared with the relatively detailed understanding of how FH2 domains track on the filament end, essentially nothing is known about how this works for VASP.

p. 8, line 5: "for" not "towards G-actin".

p. 8, end of para 1: Calculate and include in the text and in Fig. 3D the dissociation rate constants, since they are very important to the whole argument, including the first line of the next page.

p. 9, line 7: delete "already".

p. 9, para 2 and all of p. 10: This whole section could be half as long. Just summarize the strategy and results in a few sentences.

pp. 11-12, model section: Adding a model is terrific, but I question if an analytical model with Michaelis-Menten simplifications like this one is appropriate for such a complicated reaction. The folks who modeled the formin mechanism used models with all of the reactions spelled out. This model leaves out many important reactions, including translocation of the complex on the end of the filament, dissociation of the GAB from the barbed end and the contribution of free actin monomers from the bulk phase. It also makes an assumption that actin binding to GAB is a rapid equilibrium so

that the only the equilibrium constant is considered (even though the authors measured the rate constants). A more detailed model is not difficult to formulate or simulate with freely available software and would allow a more nuanced exploration of whether the model can account for various types of data beyond the fits shown in Fig. 4E. I would start with a complete model including all of the reactions such as subunit addition from the bulk phase (rather than putting this at the bottom of p. 12) and then justify why some reactions are not included in the modeling of certain types of experiments.

I do not understand how "one VASP subunit of the tetramer binds tightly to the terminal subunit by its GAB" when the VASP complex must translocate somehow as the filament grows.

Readers would find using A for actin simpler than C.

What are the values of kt derived from the modeling? Are they faster than actin binding to GAB?

Fig. 4E does not provide a very rigorous test for the model, since the range of actin concentrations is very limited. Contrary to the text, none of these curves are anywhere near saturation. The speculations (not justified by the available data) on all of the GABs being occupied at saturation, depend on knowing the relative rates of the transfer reaction and subunit binding from solution at high concentrations of actin. What is known about these rates? The model also needs to be tested by fitting to elongation rates over a range of VASP concentrations.

Why is it justified to assume that the transfer rate is the same for all GAB constructs? It is not true for formins.

I do not understand how a single complex bound to a fixed site near the end can deliver 70-140 subunits (0.3-0.4 μm) to a barbed end.

p. 12, line 12: specify "on rate constant for a tetramer binding a filament".

p. 14, line 4: "transition to the growing end, rate of dissociation of GAG from the barbed end, or the number..."

p. 15. You should emphasize again here the similarities with formins: rate-limiting binding of subunits from solution to multiple sites on the protein (formin or VASP) followed by rapid transfer to the barbed end and rapid dissociation of the protein that delivered the new subunit.

p. 16: I would be more candid about the fact that compared with the relatively detailed understanding of how FH2 domains track on the filament end (based on crystal structures and single molecule experiments), essentially nothing is known about how this works for VASP. I disagree that "our data imply that filament elongation is mediated by a single VASP tetramer." The limited comparison of the model with experiment is consistent with this hypothesis, but is still very far away from proving it.

The data on FAB binding to filaments must be in the results section. You need to explain how you calculated that individual FAB motifs are bound to filaments 20 or 40% of the time. Surely this depends on the concentration of FAB.

p. 17: It is over-reaching to say that "our model.... predicts that all EnaVASP proteins are potent filament elongators in vivo." Experiments may be consistent with this, but the model does not prove it.

p. 18: I think that more data are required to rule out addition of actin subunits from the bulk phase during processive elongation, so this is speculation. Why should this reaction be inactive on beads when it occurs in solution experiments such as Fig. 1?

2nd Revision - authors' response   05 November 2010

Referee #2 (Remarks to the Author):

The authors responded very constructively to the first round of review, conducted new experiments, added a mathematic analysis and rewrote parts of the paper. Overall, the revised ms is far better than the original. Some of the new parts of the paper still need some refinement.

p. 5, final paragraph of introduction: After explaining some fundamental differences between formins and VASP, the final conclusion regarding the mechanism for delivery of actin subunits to the barbed end of filaments is remarkably like the formin mechanism, i.e. rate-limiting binding of subunits from solution to multiple sites on the protein (formin or VASP) followed by rapid transfer to the barbed end and rapid dissociation of the protein that delivered the new subunit. This might be noted. But then I would say that compared with the relatively detailed understanding of how FH2 domains track on the filament end, essentially nothing is known about how this works for VASP.

We feel that it would be better to keep the final conclusions about the mechanism employed by VASP for the discussion section instead of anticipating the results of our work and a detailed comparison to formins. We do however state that virtually nothing is known about the molecular details of VASP-mediated filament elongation (top of page 5).

p. 8, line 5: "for" not "towards G-actin".

Thanks. This has been changed.

p. 8, end of para 1: Calculate and include in the text and in Fig. 3D the dissociation rate constants, since they are very important to the whole argument, including the first line of the next page.

The values of the dissociation rate constants of monomers from GABs, which can be easily calculated from $k_{off} = k_{on} * K_D$, are not critical to the mechanism or our arguments. Our model suggests that rapid dissociation of the GAB occurs from the monomer after its incorporation into the filament. The dissociation rate from F-actin is likely not the same than from monomers.

p. 9, line 7: delete "already".

This has been changed.

p. 9, para 2 and all of p. 10: This whole section could be half as long. Just summarize the strategy and results in a few sentences.

We followed the suggestion of the reviewer and shortened the section.

pp. 11-12, model section: Adding a model is terrific, but I question if an analytical model with Michaelis-Menten simplifications like this one is appropriate for such a complicated reaction. The folks who modeled the formin mechanism used models with all of the reactions spelled out. This model leaves out many important reactions, including translocation of the complex on the end of the filament, dissociation of the GAB from the barbed end and the contribution of free actin monomers from the bulk phase. It also makes an assumption that actin binding to GAB is a rapid equilibrium so that the only the equilibrium constant is considered (even though the authors measured the rate constants). A more detailed model is not difficult to formulate or simulate with freely available software and would allow a more nuanced exploration of whether the model can account for various types of data beyond the fits shown in Fig. 4E. I would start with a complete model including all of the reactions such as subunit addition from the bulk phase (rather than putting this at the bottom of p. 12) and then justify why some reactions are not included in the modeling of certain types of experiments.

We do not claim to have elucidated the detailed mechanism of VASP processivity required for it to be modelled to the level of detail as has been done for formins, i.e. accounting for various intermediates states and transitions. As the reviewer indicates, relatively little is known about the VASP mechanism, but our paper does offer important new insights. The advantages of our model are that it is simple, digestible to most readers of EMBO J, and accurately predicts the elongation rates with very few fitted parameters, and yet it still accounts for the key steps in the proposed mechanism (monomer binding to GAB, and transfer of the monomer to the filament coupled to

rapid GAB release). It also quantitatively explains how affinity can enhance elongation rates in a saturating dependence on monomer concentrations. Addition of further complexities to the model is simply not justified at this point and would violate the principle of parsimony.

Contrary to the reviewer's statement, our model assumptions do not include rapid equilibrium of GAB-monomer binding. This model does in fact account for the binding and dissociation rates (see Eq. 1) , and the on-rate constant kon does appear in the rate equation (Eq.2) along with the off-rate constant, replaced by $K_D = k_{off}/k_{on}$.

I do not understand how "one VASP subunit of the tetramer binds tightly to the terminal subunit by its GAB" when the VASP complex must translocate somehow as the filament grows.

Translocation is achieved upon binding a new terminal subunit and release of GAB from the penultimate subunit (previous terminal subunit). Hence, there is no separate translocation step required by this mechanism.

Readers would find using A for actin simpler than C.

We prefer to keep *C*, which we feel is equally simple and a common notation for concentration.

What are the values of kt derived from the modeling? Are they faster than actin binding to GAB?

The transfer rate constant, k_t, is a fitted parameter of the model and reported in Fig 4. Its value is determined by how fast the GAB-tethered monomer finds and binds to the tip, which should depend primarily on EVH2 size/flexibility and monomer-filament interactions, not GAB-monomer affinity. Hence, there is no obvious reason why k_t should vary among the different proteins in 4E (which merely differ in their GAB motifs). On the other hand, k_t apparently is different between DdVASP and hVASP, which we speculate is due to the difference in FAB affinity as filament-binding of EVH2 is anticipated to hinder the ability of EVH2 to transfer its monomer (see Role of FAB Motif section).

Fig. 4E does not provide a very rigorous test for the model, since the range of actin concentrations is very limited. Contrary to the text, none of these curves are anywhere near saturation. The speculations (not justified by the available data) on all of the GABs being occupied at saturation, depend on knowing the relative rates of the transfer reaction and subunit binding from solution at high concentrations of actin. What is known about these rates? The model also needs to be tested by fitting to elongation rates over a range of VASP concentrations.

We did not intend to imply that the GAB sites were fully (100%) saturated, only that they appear to be approaching saturation (i.e. saturating), which is predicted by the model. The rates the reviewer requests are k_t and $k_{on}*C$, respectively, from the model, and the parameter values are reported in the paper. There is no VASP in solution for the data in 4E – only on the beads.

Why is it justified to assume that the transfer rate is the same for all GAB constructs? It is not true for formins.

There is no mechanistic reason for the values of k_t to necessarily differ for the reasons mentioned above. Also, assuming them to be equal yields good fit to the data. Fitting individual values of k_t for each protein and fixing N=3 yields very similar values (37-43 s-1). These fits are now included in a new Supplementary Figure (Supplementary Figure 5) to make this point more clear.

I do not understand how a single complex bound to a fixed site near the end can deliver 70-140 subunits (0.3-0.4 μm) to a barbed end.

We changed the wording in the text and made clear that the tetramer is *processively* associated with the barbed end while it elongates instead of being bound to a fixed site.

p. 12, line 12: specify "on rate constant for a tetramer binding a filament".

This has been done.

p. 14, line 4: "transition to the growing end, rate of dissociation of GAG from the barbed end, or the number..."

It is not clear to us what the reviewer is referring to here.

p. 15. You should emphasize again here the similarities with formins: rate-limiting binding of subunits from solution to multiple sites on the protein (formin or VASP) followed by rapid transfer to the barbed end and rapid dissociation of the protein that delivered the new subunit.

We feel the present discussion of this mechanism in the general context of other filament end-tracking proteins suffices.

p. 16: I would be more candid about the fact that compared with the relatively detailed understanding of how FH2 domains track on the filament end (based on crystal structures and single molecule experiments), essentially nothing is known about how this works for VASP. I disagree that "our data imply that filament elongation is mediated by a single VASP tetramer." The limited comparison of the model with experiment is consistent with this hypothesis, but is still very far away from proving it.

We changed "imply" to "strongly suggest".

The data on FAB binding to filaments must be in the results section. You need to explain how you calculated that individual FAB motifs are bound to filaments 20 or 40% of the time. Surely this depends on the concentration of FAB.

We moved the data on the role of the FAB motif to the results section. The explanation of the calculation is
given in the in Supplementary Information.

p. 17: It is over-reaching to say that "our model.... predicts that all EnaVASP proteins are potent filament elongators in vivo." Experiments may be consistent with this, but the model does not prove it.

We did not intend to state and neither wrote that it is **proven** that all Ena/VASP proteins are potent filament elongators *in vivo*. Notwithstanding this, models are generally used to provide predictions However, in case thewording was too strong, we changed "predicts" to "anticipates".

p. 18: I think that more data are required to rule out addition of actin subunits from the bulk phase during processive elongation, so this is speculation. Why should this reaction be inactive on beads when it occurs in solution experiments such as Fig. 1?

It may have been too strong to state that the direct pathway is entirely inhibited when VASP is coated on beads. However, besides the explanations that are already given in the text and supplementary Figure 6, calculations made by employing our model also strongly favour our interpretation that the direct pathway is negligible on beads: Firstly, if the direct pathway would still occur with comparable rates as with VASP in solution ($\sim 8\mu M^{-1}s^{-1}$), the elongation-data could not be fitted with the same kt-values for solution-phase and clustered. Secondly, fitting of the elongation rates mediated by VASP-proteins clustered on beads under consideration of the direct pathway yielded a very low value for k_f of $0-0.25 \mu M^{-1}s^{-1}$ (and an only marginally better fit compared to fits that do not consider the direct pathway), again showing that the direct pathway is at least greatly hampered when VASP is clustered on beads. Since we feel that this is an important point that needs to be addressed in more detail, we included these finding in the results section and also present an additional supplementary figure (Supplementary Figure 5), showing the fits to the experimental data. With this additional information the difference between VASP-mediated filament elongation in solution and on beads is much clearer. In order to avoid a too strong wording, we also changed respective sections in the manuscript.

4th Editorial Decision   29 November 2010

Thank you for sending us your re-revised manuscript. We have now received the comments of referee 2 who expresses disappointment regarding the revisions made (see below). Now, I will not insist on expanding the study along these lines, and the manuscript will therefore be publishable in The EMBO Journal. Still, I would like to ask you to take his/her comments into account and at least respond to those once more. Furthermore, I need to ask you to discuss the JCB article by Hansen & Mullins (PMID: 21041447) that has been published in the meantime in the discussion section. Finally, I would like to urge you to go once more through the manuscript text, ideally with assistance by a colleague outside the field, to make it more easily accessible to the non-specialist reader.

Please let us have an amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

This second round of revision moved this important paper closer to acceptance, but the authors were stubborn about not taking some well-meaning advice on how to strengthen their paper. At this point the editor will have to decide if the changes that I suggested have merit or if they want to publish the paper as it stands.

Here are the points were we disagree.

p. 8, end of para 1: Calculate and include in the text and in Fig. 3D the dissociation rate constants, since they are very important to the whole argument, including the first line of the next page. The authors replied that the values of the dissociation rate constants of monomers from GABs, which can be easily calculated from $koff = kon*KD$, are not critical to the mechanism or our arguments. Our model suggests that rapid dissociation of the GAB occurs from the monomer after its incorporation into the filament. The dissociation rate from F-actin is likely not the same than from monomers. The authors should have taken this simple suggestion, since the dissociation rate constant from monomers is informative.

pp. 11-12, model section: I suggest a model with all of the reactions spelled out, as done for formins. The authors replied that since relatively little is known about the VASP mechanism, their simple model should suffice and that "addition of further complexities to the model is simply not justified at this point." I disagree. They would be better off with a better model without assumptions that are difficult to verity.

Fig. 4E: I was concerned that none of these curves are anywhere near saturation in spite of claims in the text. The authors replied that they "did not intend to imply that the GAB sites were fully (100%) saturated, only that they appear to be approaching saturation (i.e. saturating), which is predicted by the model. In spite of my admonition the text still says: "with an apparent saturating dependence on monomer concentration". One must get onto the plateau to demonstrate saturation. Anything short of the plateau simple tells one nothing about the saturation value.

p. 14, line 4: "transition to the growing end, rate of dissociation of GAG from the barbed end, or the number..." The authors replied that it is not clear to us what the reviewer is referring to here. I suggested that they include the phrase "rate of dissociation of GAG from the barbed end" to this sentence.

3rd Revision - authors' response   01 December 2010

Referee #2 (Remarks to the Author):

Breitsprecher et al. EMBO J revised EMBOJ-2010-74730R3

Although we have not agreed on all points, we are very grateful for the reviewer's helpful comments which have improved our paper substantially.

p. 8, end of para 1: Calculate and include in the text and in Fig. 3D the dissociation rate constants, since they are very important to the whole argument, including the first line of the next page. The authors replied that the values of the dissociation rate constants of monomers from GABs, which can be easily calculated from koff = kon*KD, are not critical to the mechanism or our arguments. Our model suggests that rapid dissociation of the GAB occurs from the monomer after its incorporation into the filament. The dissociation rate from F-actin is likely not the same than from monomers. The authors should have taken this simple suggestion, since the dissociation rate constant from monomers is informative.

We have considered this point and now report in Fig. 4F the values of koff that we calculate from $k_{on}*K_D$.

pp. 11-12, model section: I suggest a model with all of the reactions spelled out, as done for formins. The authors replied that since relatively little is known about the VASP mechanism, their simple model should suffice and that "addition of further complexities to the model is simply not justified at this point." I disagree. They would be better off with a better model without assumptions that are difficult to verity.

We respect the fact that the reviewer has a different position on this, but the key purpose of our model is to quantitatively explain the different observed rates for the different proteins of different monomerbinding affinities. For this, it is sufficient to assume binding of monomers to GAB and an irreversible transfer of the monomers to the filament tip. Stipulating the steps required for GAB to be subsequently released from the filament is not required for our model to be valid and make useful predictions, so long as GAB release is sufficiently fast to maintain N=3 by quickly freeing up GABs for further monomer binding. Our data do not discern the multiple possibilities for GAB release, which include, for example, rapid first-order release from all non-terminal subunits following monomer transfer, concurrent release of GAB coupled with the monomer transfer step, or perhaps some step involving ATP hydrolysis. This primary purpose of this model, which is to explain why affinity enhances elongation, is met without requiring the as-of-yet unknown other steps in the end-tracking cycle."

Fig. 4E: I was concerned that none of these curves are anywhere near saturation in spite of claims in the text. The authors replied that they "did not intend to imply that the GAB sites were fully (100%) saturated, only that they appear to be approaching saturation (i.e. saturating), which is predicted by the model. In spite of my admonition the text still says: "with an apparent saturating dependence on monomer concentration". One must get onto the plateau to demonstrate saturation. Anything short of the plateau simple tells one nothing about the saturation value.

We think most readers would recognize that the dependence can be "apparently saturating" (i.e. appearing to level off) without having yet reached full saturation (i.e. at a plateau). But in case the wording was too strong, we changed the phrase and replaced "..apparent saturating dependence" by "…appear to be approaching saturation".

p. 14, line 4: "transition to the growing end, rate of dissociation of GAG from the barbed end, or the number..." The authors replied that it is not clear to us what the reviewer is referring to here. I suggested that they include the phrase "rate of dissociation of GAG from the barbed end" to this sentence.

According to the suggestion of the reviewer we reworded the phrase to "...kinetics of monomer transfer and GAB release from the barbed end, or the number...".